

The Pesticide Malathion Disrupts *Xenopus* and Zebrafish Embryogenesis: An Investigative Laboratory Exercise in Developmental Toxicology

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Abstract: Malathion is an organophosphorus insecticide, which is often sprayed to control mosquitoes. When applied to aquatic habitats, malathion can also influence the embryogenesis of non-target organisms such as frogs and fish. We modified the frog embryo teratogen assay in *Xenopus* (FETAX), a standard toxicological assay, into an investigative undergraduate laboratory exercise. This exercise provided students with experience in developmental toxicology, experimental design, quantitative morphology, digital imaging, and presentation of research results. Their results demonstrated that *Xenopus* embryos exposed to malathion on the first or second day of development were indistinguishable from controls, while embryos exposed to malathion on the third day of development exhibited significantly bent tails and shorter body lengths. Similarly, sublethal malathion exposures also compromised early zebrafish development. To determine if this investigative laboratory exercise met its goal of fostering conceptual understanding of developmental toxicology, we compared student performance on a questionnaire before and after the laboratory exercise, which demonstrated significant improvement in conceptual understanding. Moreover, all (45/45) students successfully completed a modified FETAX and prepared posters of their results, indicating that students learned quantitative morphology and imaging skills while also gaining valuable experience in designing, executing, and communicating an experiment.

Keywords: undergraduate, *Xenopus*, zebrafish, malathion, developmental biology, environmental biology, laboratory exercises, toxicology

Introduction

Development is a critically sensitive period where changes in environmental conditions can alter the normal program of embryogenesis (Gilbert, 2001). The limb malformations of children exposed to thalidomide (Newman, 1986; Stephens and Brynner, 2001), cognitive difficulties in children with fetal alcohol syndrome (Dorris, 1989; Mattson *et al.*, 2001), and concerns when retinoids are used as acne treatments for women of child-bearing ages (Lammer *et al.*, 1985; Ross *et al.*, 2000) provide dramatic evidence for the existence of sensitivity to external factors and critical periods in human embryogenesis. Such striking examples of windows of sensitivity to teratogens often capture considerable attention and interest from undergraduate students.

Amphibian and fish embryogenesis are sensitive to environmental factors including temperature, pH, nutrient levels, or chemicals such as pesticides. Malathion, an organophosphorus (OP) insecticide, is frequently sprayed on aquatic habitats and crops to

control soft-bodied insects such as mosquitoes and boll weevils. Approved for residential, agricultural, and public health uses, OPs account for half of the total insecticide use in the U.S. (Environmental Protection Agency, 1998). OP insecticides are particularly favored in agriculture because they are inexpensive, kill a wide variety of insects, rarely lead to resistance, and degrade relatively quickly (Kumar and Ansari, 1984; Environmental Protection Agency, 1998). Malathion, like all OPs, acts by inhibiting the enzyme acetylcholinesterase (AChE), which is present in cholinergic synapses and prevents excessive stimulation of postsynaptic neurons and muscles by breaking down the neurotransmitter acetylcholine (ACh) (Alam and Maughan, 1992). While malathion's toxicity is targeted to invertebrates, non-target organisms are often exposed during pest control events (Hall and Kolbe, 1980). Despite malathion's rapid degradation, even brief exposure can alter the development of non-target

animals, particularly aquatic vertebrate embryos (Cook et al., 2005). Thus, the influence of the pesticide malathion on the development of non-target, aquatic embryos is of considerable environmental relevance. Recently, vigorous debate on pesticide testing in humans has highlighted the importance of understanding how chemicals influence human health (Skokstad, 2005; Resnick and Portier, 2005).

FETAX (frog embryo teratogen assay: *Xenopus*) is a common toxicological method used to evaluate a substance's ability to disrupt normal development (Davies and Freeman, 1995). FETAX is a simple test that rears recently fertilized *Xenopus laevis* embryos in a solution containing the potential teratogen. External tadpole morphology is assessed after 96 hours of exposure and any substance is considered a teratogen if it causes significant alterations in external tadpole morphology. Because numerous mechanisms of development are conserved between vertebrate species, substances that impair *Xenopus* development may potentially impair the development of other vertebrates, including humans (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods & National Institute of Environmental Health Sciences, 2000).

Recent widespread declines in amphibian populations (Blaustien and Wake, 1990; Stuart et al., 2004) make collecting native embryos potentially threatening to local populations and ecology. As an alternative, the South African clawed frog, *Xenopus laevis*, is ideal for developmental toxicology research because *Xenopus* are commercially available, reproduce year round, produce large numbers of eggs that can be fertilized *in vitro*, and embryonic development is exceptionally well documented (Nieuwkoop and Faber, 1956; Xenbase, 2005). Similarly, the zebrafish (*Danio rerio*), a more recent model vertebrate, provides comparable advantages of rapid development, year-round fertilization, large clutch size, transparent embryos, and well-documented embryology, with the additional advantages that the genome is well characterized and many mutant and transgenic fish are readily available and amenable to developmental toxicology studies (Westerfield, 200; Grunwald and Eisen, 2002; Spitsbergen and Kent, 2003; Bradbury, 2004; Hill et al., 2005; Zebrafish Information Network, 2005).

FETAX analysis has shown that malathion exposure causes pigmentation abnormalities, severe edema, and abnormal gut and notochordal bending in tadpoles (Snawder and Chambers, 1989). Moreover, *Xenopus* embryos were specifically sensitive to malathion's teratogenic effects in discrete developmental windows (Snawder and Chambers, 1990). In order to provide undergraduates in a developmental biology course with opportunities to

examine the sensitivity of *Xenopus* embryos to malathion's teratogenic effects, we modified the FETAX into an investigative laboratory exercise. Our goals for this exercise were for students to 1) work with developing *Xenopus* embryos, 2) design a FETAX experiment, 3) execute the experiment they designed, 4) use imaging software to quantify embryo morphology, and 5) understand important concepts in developmental toxicology such as teratogens and critical windows of sensitivity. Moreover, this example of an investigative laboratory experience is consistent with current science pedagogy that encourages undergraduate courses to provide opportunities to learn science in the way science is done, specifically by encouraging students to propose and test hypotheses in quantitative ways (McNeal and D'Avanzo, 1997; National Research Council, 1997, 1999, 2000a, 2000b, 2003). This participatory, inquiry-based approach to laboratory exercises is favored over traditional "cookbook" labs, because in the design, execution, and communication of an experiment students experience the process and excitement of research methods and they have opportunities to think independently and creatively.

Methods

Xenopus laevis embryos

Xenopus laevis embryos were obtained by *in vitro* fertilization and staged visually according to pictorial staging tables (Nieuwkoop and Faber, 1956; Cebra-Thomas, 2003; Graham et al., 2005; Xenbase, 2005). Adult females (Xenopus One) were induced to superovulate by subcutaneously injecting 600 units human chorionic gonadotropin (Sigma). The next morning eggs were manually extirpated from the superovulating females into sterile plastic dishes containing a modified rearing (MR) solution (also known as 20% modified Steinberg's solution: 60 mM NaCl, 0.67 mM KCl, 0.3 mM Ca(NO₃)₂, 0.83 mM MgSO₄, 10 mM HEPES, and 40 mg/L gentamycin, pH 7.4) (Keller, 1991). Eggs were immediately mixed with a small piece of crushed testes, harvested from an adult male within the previous week, which had been stored in 5X sterile MR at 4 °C. About 30 minutes after fertilization (at room temperature) cortical rotation was observed, which was indicated by most eggs oriented with the dark (animal) pole facing up. About 90 minutes after fertilization the first cleavage occurred and the eggs were clearly divided into two symmetrical blastomeres. After several more rounds of mitosis, asymmetric blastomeres became apparent. To make embryo distribution and handling more convenient, jelly coats were removed from cleaving embryos (stages 2-8) by brief immersion in 2% (w/v) cysteine in MR just long enough until the jelly coats had dissolved (usually less than five minutes). All dejellied embryos were

then thoroughly rinsed with at least three changes of MR and placed in new plastic petri dishes. Fertilizing eggs at approximately 9:00 AM conveniently provided stage 6-8 blastulas for a laboratory session that started at 1:00 or 1:30 PM. *Xenopus* can be obtained from a variety of providers (Xenopus One, Nasco, Carolina). Helpful manuals (Carolina Biological, 1993; Sive et al., 2000) and media (Grainger and Sive, 1999; Pickett-Heaps and Pickett-Heaps, 1999; Tyler and Kozlowski, 2003) are available for more detailed information on *Xenopus* husbandry, techniques, and development. All animal procedures were approved by Davidson's Institutional Animal Care and Use Committee.

Zebrafish embryos

Adult zebrafish from a local pet store were maintained in 5 L aquaria at 27-28°C, pH range 6.8-7.5 with high dissolved oxygen levels (83-87% saturation), and a 14:10 hour light:dark photoperiod. The fish were fed a rotating diet of commercial flake food, frozen brine shrimp, and bloodworms. Eggs were obtained as described by Westerfield (2000) with modifications per Cook et al. (2005). Three to four zebrafish (at least two females and one male) were placed into homemade false-bottom plastic containers before the dark period began. When the light period began, the fish laid and fertilized eggs that fell through the false-bottom grids and collected in a container below that was inaccessible to the adults who would otherwise consume the eggs. Within two hours, adult fish were returned to the aquaria and eggs were transferred to a glass finger bowl using a plastic pipette (with the tip cut off to minimize chorion damage during transfer). Embryos were rinsed once with clean tank water and then observed under a stereomicroscope to discriminate between fertilized and unfertilized eggs. Fertilized eggs were identified by the presence of distinct blastomeres, while unfertilized eggs were often milky and lacked discernable blastomeres. Shortly after fertilization, zebrafish embryos were reared in tank water that included malathion for exposure durations lasting up to 120 hours. Helpful laboratory manuals for research (Westerfield, 2000; Nusslein-Volhard and Dahm, 2002) and teaching (Tyler, 2003) as well as websites (Zebrafish Information Network, 2005) are available for more detailed information on zebrafish care and techniques. Movies of early zebrafish development are also available (Kane and Warga, 1991; Kane and Karlstrom, n.d.; Karlstrom and Kane, 1996) on the internet as are zebrafish staging tables (Kimmel et al., 1995; Cebra-Thomas, 2001) to help students identify embryo stages and anatomical structures.

Malathion solutions

Malathion solutions, widely available to consumers in the pesticide section of most home and garden stores, are frequently sold as 50% solutions with labels indicating that the pesticide is harmful to amphibian embryos. Manufacturer's guidelines for consumer use of malathion suggest application of malathion at approximately 34 g/L. We conducted preliminary FETAX experiments using two different commercial malathion solutions diluted to 0.001% (v/v) in MR. We observed that aqueous solutions of commercial malathion stored at room temperature often formed a white precipitate and its potency changed with storage. 0.001% (v/v) solutions of commercial malathion caused obvious developmental deformities in *Xenopus* embryos, though with inconsistent efficacy (data not shown). Consequently, we recommend that instructors who wish to use commercial malathion should dilute the 50% solution immediately before use and avoid prolonged storage. Further, neither of the two commercial malathion solutions we purchased identified the constituents of the other 50% of the solution, making it impossible for students to design appropriately controlled experiments. Given the disadvantages of using commercial malathion, all results reported here were obtained with pure (99.5%) malathion from a chemical supplier (Supelco). While the cost of purified malathion was approximately \$50 more than consumer products, the pure malathion produced more consistent results, permitted longer storage of stock solutions, and allowed students to design appropriately controlled experiments. We diluted malathion in acetone to generate a stock solution of 10 g/L, which was stored at 4°C in glass bottles shielded from light with aluminum foil. Working malathion solutions were diluted in MR immediately before use with *Xenopus* or were diluted in tank water for use with zebrafish embryos.

Modified FETAX procedure

Approximately 20 properly cleaving dejellied stage 6-8 (Nieuwkoop and Faber, 1956) *Xenopus* embryos were placed in a total of 50 mL of MR solution (untreated), MR + acetone (vehicle control), or MR + malathion in 100 mm plastic Petri dishes at room temperature. To determine a window of sensitivity to malathion, groups of embryos were exposed to malathion concentrations for day 1 (0-24 hours), day 2 (24-48 hours), or day 3 (48-72 hours) of development. Sibling embryos were reared in malathion for the entire three day period (0-72 hours). All solutions were changed at 24 hour intervals to control for any effects of embryo handling. When embryos were removed from malathion they were rinsed three times with MR and

subsequently reared in a fresh dish of MR. After 72 hours tadpoles reached stages 41-42 of development (Nieuwkoop and Faber, 1956). They were anesthetized in MR containing anesthesia (0.05% (w/v) MS-222 at pH 7.4) for approximately five minutes before fixation in 1% (v/v) glutaraldehyde in phosphate buffered saline (PBS). Tadpoles were fixed at 4 °C for 1-30 hours, then rinsed thoroughly with at least four changes of PBS and stored in PBS at 4 °C until morphological analysis.

Morphological analysis

To capture digital images of each tadpole or zebrafish for quantitative morphology we used CoolPix 995 cameras (Nikon) mounted on the eyepieces of stereomicroscopes via MMCOOL adaptors (Martin Microscope, Easley, SC). The free digital imaging program, ImageJ (rsb.info.nih.gov/ij) was used to measure tadpole (or zebrafish) morphology. A best-fit midline of each embryo was manually traced along the spine using a segmented line. The axis angle was defined as the angle formed between the embryo's posterior spine and a straight line extending from the anterior spine. Similarly, the length of each embryo was determined by drawing a line along the axis of the embryo from the cement gland (anterior) to the tip of the tail (posterior).

Statistical analysis

Using Minitab software (Release 13.3 for Windows), analyses of variance (ANOVAs) were performed on the two response variables of axis angle and body length (both \log_{10} -transformed to approximate normality). The statistical model was a one-way ANOVA with the ten treatments (eight treatments and two controls) as a fixed effect and nested within trial. If a trial was determined to be non-significant, as it was for log-transformed axis angle, we combined all trials to determine overall treatment effects. This approach allowed us a more robust assessment of treatment effects, regardless of trial effects. Tukey pairwise comparisons were used to compare the average response among the ten groups. For body length, because of significant trial effects, we performed the ANOVA separately for each trial so the Tukey test could be used to determine treatment effects within a trial instead of across treatment-trial combinations, which would have been unwieldy due to forty different combinations. The alpha level was set at 0.05.

Malathion FETAX as a laboratory exercise

The malathion FETAX laboratory exercise was adapted into an investigative laboratory exercise in Biology 306 (Developmental Biology), an upper level laboratory course at Davidson College that enrolled 15 students in 2003 and 30 students in 2004, most of whom were biology majors. Laboratory

sections consisted of 6-16 students and were taught by the course instructor (BL). Students had been introduced to staging embryos with pictorial staging tables, using digital cameras on double-headed stereomicroscopes, generating scale bars and calibrating software, analyzing morphology with ImageJ, and preparing figures with Adobe PhotoShop software in previous laboratory sessions (Figure 1). In preparation for this developmental toxicology laboratory, students were required to read a brief laboratory handout that outlined the exercise and suggested avenues for experimentation (Appendix I) and a short section in their textbook on teratogens (Gilbert, 2003). At the start of the laboratory session, the instructor briefly introduced malathion's use as an insecticide, the concept of teratogens, and FETAX procedures. Students then designed experiments employing FETAX to assess malathion's influence on *Xenopus* development by developing a plan and discussing that plan with classmates and the instructor. Students were then invited to design an experiment that tested a single variable of their own choosing such as duration of treatment, embryonic stage at treatment, or pesticide concentration, *etc.* Healthy embryos at various stages of development, the 10 g/L stock solution of malathion, acetone, and MR were provided at the beginning of the laboratory period for students to use in their experiments. Students wore protective gloves and worked in a chemical fume hood while handling the acetone and malathion. Allowing students to dilute the stock malathion (and control acetone) into working concentrations provided valuable reinforcement of the practical lab skills required to prepare solutions. Because a specific goal of this exercise was to allow students to quantify morphological features of malathion-treated embryos (using techniques they had been introduced to in previous laboratory sessions), we discouraged students from using lethal concentrations of malathion in their experiments so that they would have viable embryos to measure. Sublethal malathion concentrations of 1.0-2.5 mg/L were recommended as reasonable concentrations for experimentation, though students were free to select other concentrations for their experiments. A traditional three-hour laboratory period provided ample time for the instructor to consult with each of the 6-16 students regarding experimental design and for students to set up a FETAX experiment of their own. A typical FETAX rears embryos for 96 hours, however we modified the assay to 72 hours so that weekend laboratory work was not required. Students worked alone (2003) or with a laboratory partner (2004) and were responsible for returning to the laboratory on their own time to change solutions and fix embryos as their individual experimental designs dictated (Figure 1). The subsequent scheduled laboratory period provided open working time for

students to acquire images, analyze their results, and discuss their findings. For communication and evaluation purposes, students were required to submit two color copies of a small scientific poster (8.5 x 11 inch) illustrating their experiment and results. One

copy was posted in the laboratory as a way for students to share their results with their classmates and the other copy was graded by the instructor (BL).

Pre	Scientific skills introduced in previous laboratory periods: <ul style="list-style-type: none"> • Introduction to pictorial tables to stage <i>Xenopus</i> embryos • Introduction to ImageJ to measure distances on magnified images • Introduction to using Photoshop to assemble figures & scale bars
Week 1	Formal 3-hr laboratory period <ul style="list-style-type: none"> • Experimental design (written plan & consultation) • Experiment execution (placing embryos in appropriate solutions) Students return to lab on their own time <ul style="list-style-type: none"> • Experiment execution (change solutions, fix embryos, <i>etc.</i>)
Week 2	Formal 3-hr lab period <ul style="list-style-type: none"> • Data collection (capturing digital images of embryos) <ul style="list-style-type: none"> • Data analysis (measuring embryo morphology) • Data interpretation (identifying conclusions)
Post	Scientific posters <ul style="list-style-type: none"> • Data communication (posters displayed in the laboratory) • Evaluation (posters graded by instructor)

Figure 1. General plan of the malathion developmental toxicology exercise. This investigative laboratory exercise in developmental toxicology was completed in two, traditional afternoon laboratory sessions. Basic embryo staging and digital imaging skills were introduced in an earlier laboratory session and access to the lab after hours was provided so that students could fully execute their experiments.

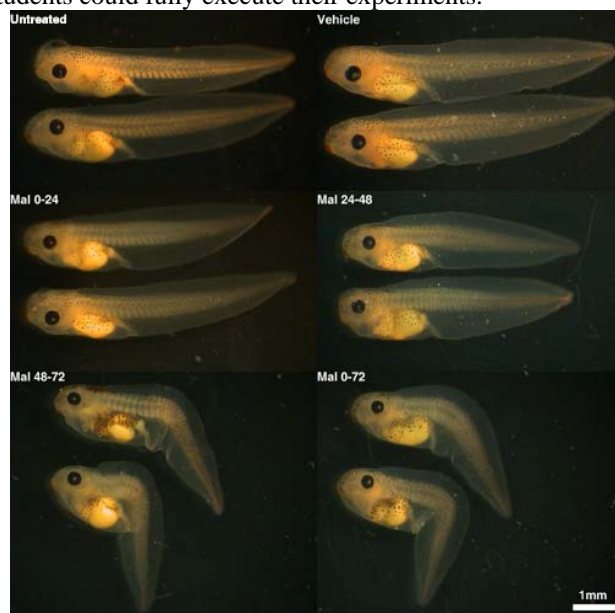


Figure 2. The pesticide malathion disrupts *Xenopus* axis development. Images show typical examples of 72 hour tadpoles representing untreated controls, control tadpoles exposed to vehicle (0.05% acetone), and tadpoles exposed to 2.5 mg/L malathion for one of the three days (0-24 hr, 24-48 hr, or 48-72 hr) or the entire three day period (0-72 hr). Note the dramatic axis deformation in the tadpoles exposed to malathion on the third day of development (Mal 48-72) or throughout the first three days of development (Mal 0-72).

Assessing the Modified FETAX Laboratory Exercise

To determine if students learned the concepts of teratogens, target vs. non-target organisms, and critical periods of development, we administered a brief questionnaire. Students completed this voluntary and ungraded questionnaire twice: first prior to any classroom discussion of teratogens, developmental toxicology, or critical periods (pre-lab) and then again approximately one week after submitting posters reporting their results (post-lab). Specifically the questionnaire asked: 1. What is a teratogen? 2. What is a target organism? 3. What is a non-target organism? 4. Compare how pesticides affect target and non-target organisms. 5. What is a sensitive period? Each questionnaire was independently scored several weeks later by two individuals (2003 – DCC and BL; 2004 – IRW and BL) who were each blind to the students' names and whether a questionnaire was completed pre- or post-lab. Each response was scored on a scale from zero to three with zero demonstrating no understanding of the question material and three demonstrating a secure understanding of the question material. The scores from the two graders were then averaged together. Paired t-tests were performed on the pre-lab versus post-lab scores using Prism (GraphPad). Assessment methods were approved by Davidson College's Human Subjects Internal Review Board.

Results

Control and malathion treated embryos reliably developed to stage 41-42 in 72 hours (Figure 2). Vehicle-treated tadpoles developed indistinguishably from control tadpoles, indicating that the small volume of acetone used to dissolve the malathion had no demonstrable effects on *Xenopus* embryogenesis. In contrast, tadpoles treated with malathion exhibited dramatically bent morphologies that were clearly visible to eyes unfamiliar with *Xenopus* embryo development (Figure 2). To evaluate the extent of axis deformation the angle of deviation between the anterior and posterior axes was measured for each tadpole. *Xenopus* tadpoles exposed to 1.0 or 2.5 mg/L malathion either on the third day of development (48-72 hours) or throughout the three-day experiment (0-72 hours) exhibited significantly bent axes ($F = 70.57$; $df = 9, 910$; $P < 0.0001$; Figure 3) and were shorter in length than control embryos ($F = 52.00$; $df = 36, 879$; $P < 0.0001$; Figure 4). Thus, the effects of malathion on external *Xenopus* embryo morphology were reliable and dramatic.

In addition to axis and length abnormalities, malathion treatment also resulted in tadpoles that did not swim as frequently or as rapidly as the controls (Chemotti and Lom, *personal observation*). When

these tadpoles with bent axes did move, they swam in circles, unlike control tadpoles that could swim in straight trajectories.

In addition to the dramatic and easily measurable effects of malathion on *Xenopus* embryos, a subset of undergraduates pursuing independent study projects demonstrated that zebrafish embryogenesis is also sensitive to similar malathion concentrations when treated for 96 hours (Figure 5). Zebrafish embryo lengths, eye diameters, abdominal areas, hatching schedule, heart rate, and AChE activity are altered by malathion exposure (Cook *et al.*, 2005; Davis, Rose, Garren, Hodge, and Lom, unpublished). Thus this laboratory exercise can be easily adapted to other aquatic vertebrate species such as zebrafish to provide novel research experiences in developmental toxicology.

To assess student learning via this malathion FETAX laboratory exercise, performance on pre-lab and post-lab questionnaires was compared (Figure 6). All students who completed both surveys scored higher on the post-lab survey than they did on the pre-lab survey, indicating that understanding of concepts such as teratogens and sensitive windows of development was enhanced after completing this investigative exercise. Paired t-tests for each question revealed that student performance was significantly improved ($P < 0.05$) after completing the laboratory exercise.

Discussion

Malathion exposure on the third day of development (via 48-72 hr or 0-72 hr exposures) induced significant and dramatic axis deformation and shortened lengths of *Xenopus* embryos. These results indicate that the third day of development in *Xenopus* is a critical window of sensitivity to malathion's teratogenic effects. While this 72 hr, modified FETAX revealed a critical window in which *Xenopus* sensitivity to malathion's teratogenic effects begins, longer experiments to determine if and when *Xenopus* tadpoles cease to be sensitive to malathion's teratogenic effects could be designed and conducted by undergraduates. Similarly students could design longer-term experiments to determine if brief, early exposure to a pesticide exerts long-term effects on tadpole morphology, physiology, or behavior.

Whereas malathion is an acetylcholinesterase (AChE) inhibitor, the mechanisms by which the pesticide causes axis deformation are not well understood, but may be related to the integrity of the extracellular matrix. The 48-72 hour sensitivity window to malathion coincides with a period of increased collagen synthesis in *Xenopus* development (Green *et al.*, 1968). Collagen makes up the notochord sheath and

malathion induces abnormalities consistent with collagen defects, including bent notochords,

circulatory defects, and shortened body lengths

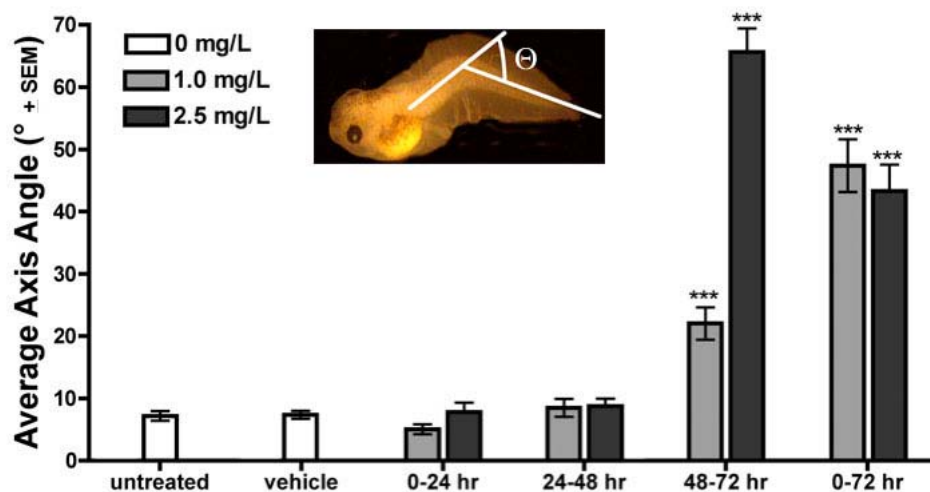


Figure 3. Malathion significantly bends the axes of developing *Xenopus*. When exposed to 1.0 or 2.5 mg/L malathion on the third day of development (48-72 hr) or for the first three days of development (0-72 hr) tadpoles axes were significantly more bent than untreated or vehicle-treated control tadpoles. Inset image demonstrates how axis angles (°) were measured. *** = $p < 0.001$ when compared to controls. N=920 tadpoles total from four separate experiments.

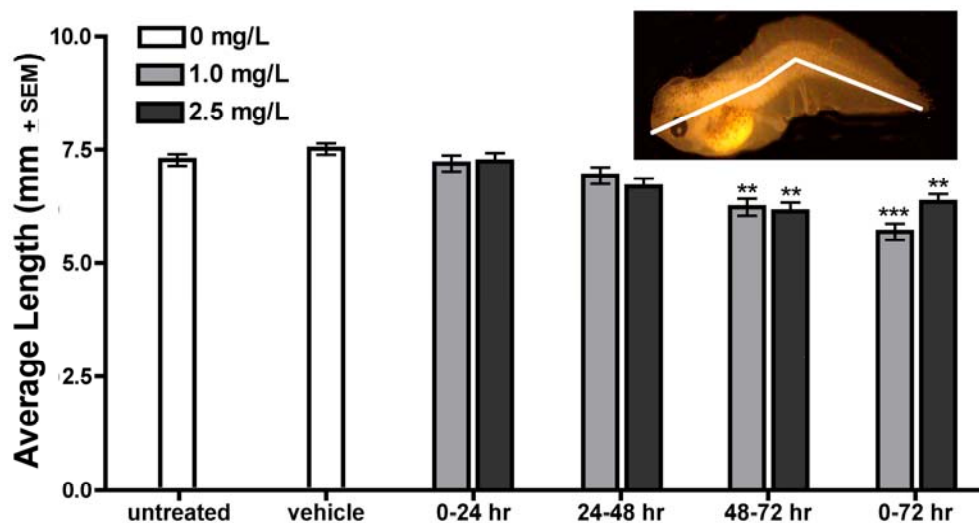


Figure 4. Malathion significantly shortens developing *Xenopus*. When exposed to 1.0 or 2.5 mg/L malathion on the third day of development (48-72 hr) or for the first three days of development (0-72 hr) tadpoles were significantly shorter than untreated or vehicle-treated controls. Inset image demonstrates how lengths were measured. ** = $p < 0.01$, *** = $p < 0.001$ when compared to controls. N=920 tadpoles total from four separate experiments.

(Snawder and Chambers, 1990, 1993). Thus, disruptions of the notochord sheath may contribute to abnormal bending and/or shortening of the embryo. Additionally, malathion affects ascorbic

acid, hydroxyproline, lysyl oxidase, and NAD^+ (Snawder and Chambers, 1989, 1993). Proper hydroxyproline levels are necessary for the formation of collagen's triple helix, which allows fibroblasts to release collagen (Deyl and Adam, 1989). In



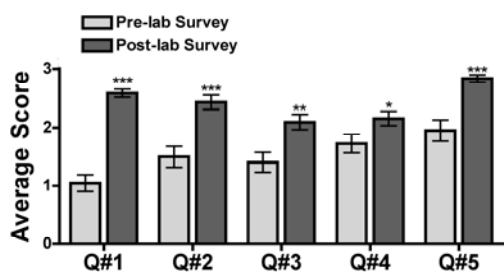
Figure 5. Malathion also alters zebrafish axis development. Undergraduates can also apply FETAX methodologies to zebrafish embryos. Images show typical examples of 96 hour zebrafish representing control embryos continuously exposed to vehicle (0.05% acetone), or 2.5 mg/L malathion for 96 hours. Similar to results with *Xenopus*, pesticide-treated zebrafish embryos exhibited axis deformations, shorter lengths, and smaller eyes (see Cook *et al.*, 2005).

Xenopus, malathion reduces ascorbic acid and hydroxyproline levels and inhibits collagen proline hydroxylase, resulting in a reduced number of extracellular collagen fibers. Even though total collagen amounts are unaffected, this may suggest that malathion may target collagen extracellular transport and crosslinking (Snawder and Chambers, 1993). Since lysyl oxidase aids in proper extracellular collagen cross-linking and is more sensitive to malathion than proline hydroxylase, malathion's axis deformation may be a result of reduced lysyl oxidase activity (Snawder and Chambers, 1993).

The concentrations of malathion employed in this modified FETAX (1.0 and 2.5 mg/L) are lower than recommended malathion application concentrations (estimated at 34 g/L per manufacturer's guidelines), and higher than aquatic malathion concentrations detected after application. Reported malathion concentrations in a shallow wetland ranged from <0.002 - 0.015 mg/L after malathion spraying for mosquito control (Fordham *et al.*, 2001). Lower concentrations (<0.00016 mg/L) were detected in running water of an urban stream (Kimbrough and Little, 1996). While environmental malathion concentrations are lower than the malathion concentrations tested in this exercise, several studies have shown that higher concentrations

of organophosphates can occur in small, shallow ponds, which are prime breeding habitats for amphibians (Berrill *et al.*, 1994, 1995; Fordham *et al.*, 2001). Moreover, pulses of higher pesticide concentrations are likely to occur immediately after application; higher concentrations of malathion can persist in the water for a few weeks following a single mosquito control event (Eichelberger and Lichtenberg, 1971) or for several months when malathion is sprayed more regularly (Fordham *et al.*, 2001). Because malathion is often used in agricultural settings, the resident non-target organisms of neighboring wetlands could be subject to pesticide exposure that is sufficient to compromise embryogenesis.

Tadpoles that develop a bent body axis can suffer severe consequences. A bent axis impedes normal locomotion, which in turn can limit ability to reach food sources and increase the risks of predation and desiccation. While effects beyond 72 hrs of exposure (including metamorphosis) were not examined in this report, it is likely that tadpoles with bent axes could experience impaired locomotion, feeding, and/or difficulty during metamorphosis. Additional examination of other morphological parameters (Altig *et al.*, n.d.), survival, metamorphosis, and/or locomotion in malathion-treated tadpoles provide additional open questions that could readily be addressed by undergraduates as a part of an expanded version of this laboratory



- Q#1 What is a teratogen?**
Q#2 What is a target organism?
Q#3 What is a non-target organism?
Q#4 Compare how pesticides affect target & non-target organisms.
Q#5 What is a sensitive period?

Figure 6. Students mastered concepts in developmental toxicology. Comparing average scores of pre- and post-lab questionnaires demonstrate that students scored significantly higher in the post-lab survey for each question. Thus by conducting a modified FETAX with malathion, students increased their understanding of important concepts such as teratogens, target organisms, and sensitive periods. Error bars = SEM. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. N = 45 students.

exercise or through independent study situations. We found that many undergraduates were inspired by this 72 hour FETAX experiment to pursue, related studies later in the semester via more open-ended and longer (seven week) experiments of their own design (that did not require students to execute FETAX or study pesticides). Examples of such student projects included: investigating the influence of malathion on heart rate in older *Xenopus* embryos, comparing the effects of malathion on *Xenopus* development with other pesticides such as parathion and chlorpyrifos, examining the influence of malathion on zebrafish development, the influence of malathion on zebrafish heart morphology and rate, and examining the influence of malathion on *Planaria* regeneration. Moreover, many students used their FETAX experience to design more in-depth experiments that investigated the effects of a wide variety of potential teratogens such as ethanol, caffeine, retinoids, *etc.* on the development of tadpoles, fish, and chick. Such investigations of teratogens on organisms at various stages of development and regeneration provide both compelling and practical undergraduate research experiences (Cruz, 1993; Keller et al., 1999; Johnson, 2001; Gibbs, 2003; Tyler, 2003; Mari-Beffa and Knight, 2005).

This modified FETAX assay successfully attained its objectives of providing students with an investigative laboratory experience in which they: 1) worked with developing *Xenopus* embryos, 2) had significant control over experimental design, 3) had full control over experimental execution, 4) used imaging software to quantify embryo morphology, and 5) had first-hand opportunities to understand the concepts of teratogens and critical windows of sensitivity. 100% (45/45) of the undergraduates successfully designed and executed their own, appropriately controlled variation of a FETAX. Their scientific posters (Appendix II) demonstrated that they had acquired skills to: work successfully with *Xenopus* embryos (aim 1), design experiments (aim 2), execute the experiments (aim 3), quantify embryo morphology (aim 4), and communicate their results (aim 4). Comparing responses on pre-lab and post-lab questionnaires provided a means of evaluating the effectiveness of the modified FETAX to teach concepts in developmental toxicology (aim 5; Figure 6). Student responses after the laboratory demonstrated significantly improved knowledge of teratogens, target organisms, non-target organisms, and sensitive periods. Students scored only slightly better in response to the question asking them to compare the effects of pesticides on target versus non-target organisms. The less dramatic improvement on this particular topic may result from the fact that students did not directly examine the effects of malathion on invertebrates or the question may have been obvious given the preceding two questions. The higher pre-test score on this question favors the latter possibility. Finally, this investigative FETAX laboratory exercise served as a practical and useful introduction to scientific design and execution in a developmental biology course. Later in the semester all undergraduates (45/45) successfully proposed more substantial experiments of their own design (experimental questions, controls, and organisms unspecified by the instructor) that they then conducted, analyzed, and reported to their classmates. Qualitative, end-of-semester evaluations consistently remarked that such investigative opportunities provided useful educational experiences that allowed students to develop creativity, confidence, and a practical appreciation for the process of performing and communicating scientific experiments. In summary, the modified malathion FETAX laboratory exercise described here allows students to design, execute, and analyze an experiment and evaluate quantifiable measures of vertebrate development in the larger context of ecotoxicology and teratology.

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Appendix I

FETAX LABORATORY GUIDELINES

<http://www.bio.davidson.edu/biology/balom/306/teratogenLab.html>

Background:

Normal embryogenesis can be disrupted by environmental factors that result in physically deformed embryos. In many cases embryos are more or less susceptible to teratogens at specific stages. Periods of increased susceptibility are known as critical (or sensitive) periods. Any agent that causes embryonic malformations is classified as a teratogen. The Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) is often used as a means to assess the potential teratogenicity (and toxicity) of water-soluble agents. FETAX is also used to test water quality. This week in lab you will design a modified FETAX assay to test the teratogenicity of malathion.

Laboratory Objectives:

- to become familiar with concepts in developmental toxicology
- to determine the influence of malathion on *Xenopus* development by designing, conducting, analyzing, and communicating an experiment
- to design a meaningful experiment that includes appropriate controls
- to conduct a FETAX assay with careful attention to detail and develop an awareness of tadpole morphology
- to learn how to stage *Xenopus* embryos using staging tables
- to learn how to use digital cameras to image embryos
- to learn how to quantify morphological abnormalities using ImageJ software
- to learn how to document and communicate results in both images and graphs
- to communicate your experiment via a scientific poster

Assigned reading (to be completed before you come to lab):

Gilbert (2003) pp. 740-745

Safety:

- Wear gloves when working with potential teratogens, particularly the malathion.
- The modified rearing (MR) solution contains a tiny amount of the antibiotic gentamycin. If you have allergies to this antibiotic, please wear gloves.
- Always wear gloves when working with fixatives such as glutaraldehyde. Bottles of fixative should only be opened in the chemical fume hood.
- Always mark your bottles, petri dishes, etc. with the concentration of the chemical contained therein and fill out an "experiment in progress" sheet.

Experimental Procedures:

- You may choose to vary the concentration of the teratogen, the duration of exposure, the age at

which the exposure begins, or any other parameter that is reasonable to test. You may or may not have a range of tadpole ages available at the start of lab.

- Prepare solutions in plastic vials (with caps) so you can mix solutions thoroughly by shaking. Mix solutions immediately before use.
- Use a minimum of 12 embryos per petri dish. Take time to select similarly aged embryos without malformations at the onset of your experiment.
- Check on your embryos regularly (daily recommended). If an embryo dies remove the dead embryo and record the approximate stage of death in your notes.
- Fix embryos 72 hours after starting your experiment. Transfer anesthetized tadpoles to a glass fix vial, in 2 ml of 1% glutaraldehyde for at least one hour (glutaraldehyde is a powerful fixative - always WEAR GLOVES and WORK IN THE FUME HOOD when working with glutaraldehyde). Fixed embryos must then be rinsed thoroughly (>4x) with PBS. All liquid waste must be discarded in the glutaraldehyde waste containers in the fume hood, and only fix tools (marked with black electrical tape) should be used to manipulate fixed embryos. Fixed embryos should be stored in the fix frig (marked with a large X in black tape).

Malathion

- Concentrations of either 1 mg/L or 2.5 mg/L are recommended, but you are welcome to use other concentrations. The following are directions for preparing approximately 50 ml of solution. You will use 10 ml for each 60 mm petri dish or 50 ml for each 100 mm petri dish.
 - 1 mg/L = 5 ul of 1% malathion stock in 50 ml MR
 - 2.5 mg/L = 12.5 ul of 1% malathion stock in 50 ml MR
 - Control = 5 ul or 12.5 ul of acetone in 50 ml MR

Quantifying abnormalities:

Think about how you will quantify abnormalities in your embryos. Make sure you analyze abnormalities in your embryos by at least two distinct measures. Percentage of abnormal embryos per group is one obvious and easy measure, but it is subjective. You may want to categorize abnormalities generally (*i.e.* head deformities, ventral deformities, axis deformities, *etc.*) or more specifically (reduced eye, missing tail, secondary axis, *etc.*), depending on what you observe. You must include at least one method of comparing embryos that employs ImageJ to measure some aspect of embryo morphology (*i.e.* embryo length, eye diameter, gut area, *etc.*). You can expect to have SOME time during lab next week to get assistance measuring embryo morphologies, but

you should also expect to spend some time outside of our formal lab time measuring your embryos.

Poster Assignment:

- Generate a mini poster presentation of your experiment & results.
- The poster must include a descriptive title that indicates the outcome of the experiments.
- The poster must include sections titled Introduction, Methods, Results, Summary & Conclusion, & References (with at least two references).
- Posters should be 8.5 x 11 (landscape orientation).
- Use one sans serif font consistently throughout your poster (*i.e.* Helvetica, Gil Sans, or Arial not Times or New York).
- Use bullet points to keep your statements concise (complete sentences are not necessary).
- Digital images of at least one representative embryo from each condition must be included in the poster. An accurate scale bar must be included and labeled appropriately.
- At least two graphical representations of your data must be included in the poster and one of these graphs must come from a method of analysis that employed ImageJ.
- No "raw" data allowed in the poster & always avoid tables if possible.
- Think carefully about the best graphic and visual representations of your data - try to convey your results as simply and as directly as possible - use pictures to tell your story.

- Strive to make your poster as reader-friendly as possible with simple, well-labeled figures.
- Make sure each figure includes a legend (figures do not need to be numbered in a poster).
- Each figure legend should have a title in bold that indicates the main point of your figure. The legend should be at least two sentences that briefly explains the basics of the experiment as well as defines any symbols, abbreviations, *etc.* used in the figure.
- Make sure your poster is well labeled - sample size (n), axes, scale bars, *etc.* should all be indicated.
- Submit two color printouts and email your PowerPoint file to the instructor by the deadline indicated on the syllabus.

Hints & Helpful Resources:

- The Nieuwkoop & Faber staging table diagrams of *Xenopus* development can be found at www.xenbase.org/atlas/NF/NF-all.html
- Will Graham, '02 created a photographic atlas of *Xenopus* stages that can be found at: www.bio.davidson.edu/xenopus
- A sample poster template (in PowerPoint) is available on the course's Blackboard site (look in the course material section)
- A black binder in the lab contains copies of several research articles describing the influence of malathion on embryos (do not feel limited to these articles, but feel free to use other peer-reviewed journal sources as well)

Appendix II

TWO EXAMPLES OF STUDENT POSTERS

Malathion Exposure Decreases Length and Increases Axis Angle During the 48-72 Hour Period of Development in *Xenopus laevis* Embryos

Introduction:

•Teratogens are substances that disrupt normal development and cause physical deformities in embryos.

•The Frog Embryo Teratogen Assay: *Xenopus* (FETAX) is used to determine the teratogenicity of different compounds.

•Malathion is an organophosphate pesticide used for mosquito control and in the Boll Weevil Eradication Program.¹

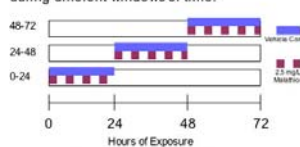
•Malathion has been shown to cause abnormal gut formation, reduced size, abnormal pigmentation, and bent notochord in *Xenopus laevis* embryos.²

•Are *Xenopus* embryos sensitive to malathion's teratogenic effects during a particular time period?

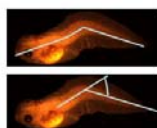
Methods:

•Fifteen stage 11 *Xenopus* embryos per condition.

•Tadpoles placed in 2.5 mg/L malathion or a vehicle control (0.025% acetone) solution during different windows of time:

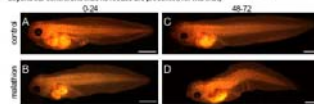


•When not in test solutions, embryos were in 20% Steinberg's solution.
•Embryos fixed in 1% glutaraldehyde after 72 hours

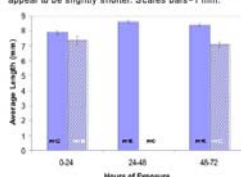


Results:

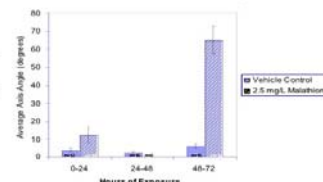
(Please note that tadpoles from the 24-48 malathion exposure were destroyed by conditions beyond our control and thus no results are presented for this time)



Malathion causes visible deformities in *Xenopus* embryos exposed on day 3 (48-72 hours). Tadpoles from acetone controls (A, C) developed normally, regardless of the period of exposure (A=0-24; C=48-72). Tadpoles exposed to malathion during the first 24 hours (B) show a slight bend in their tails. Tadpoles exposed to malathion during the 48-72 hour period, however, have a sharply bent axis and appear to be slightly shorter. Scale bars=1 mm.



Tadpole length is decreased by malathion if applied during the 48-72 hour period. The average length of the tadpoles in each of the trials was calculated. Only when malathion was applied during the 48-72 hour period did tadpoles show a statistically significant decrease in length ($p=0.007$). Note that when malathion was applied during the 0-24 hour period, the tadpoles appeared to be shorter, but the difference was not statistically significant ($p=0.120$). Bars = standard error of the mean.



Tadpoles are bent when malathion is applied during the 48-72 hour period. The average angles were computed and compared for each trial. Note that when malathion was applied during the 0-24 hour period, the tadpoles experienced a slightly greater bend in the tail than the acetone control, although the difference was not statistically significant ($p=0.064$). Also note the large increase in angle between tadpoles in the 0-24 malathion trial and the 48-72 trial. Only when malathion was applied during the 48-72 hour period did the tadpoles show a statistically significant increase in the angles of their bent tails ($p=7.72E-9$). Bars = standard error of the mean.

Summary & Conclusion:

•Malathion applied during the 0-24 hour period caused a slight, but not statistically significant, decrease in *Xenopus* tadpole length (~7% shorter) and increase in axis angle (~215% greater).

•Malathion applied during the 48-72 hour period caused a significant decrease in length (~16% shorter) and increase in axis angle (~940% greater).

➤ Malathion causes severe developmental defects if applied during the critical period of 48-72 hours in *Xenopus laevis* embryos.

References:

¹<http://www.epa.gov/pesticides/op/malathion/summary.htm> (2000).
²Snawder and Chambers. (1990) *J. Life Sci.* 46:1635-1642.

Malathion exposure results in increased body axis angle and decreased length in stage 42 *Xenopus* embryos

Introduction:

• Malathion is an aliphatic organophosphate that was introduced as an insecticide in 1950.¹

• Previous studies suggest that malathion alters posttranslational modification of collagen resulting in morphological defects in *Xenopus* embryos.²

How do different concentrations of malathion affect the morphology of developing *Xenopus*?

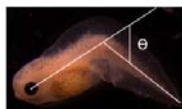
Methods:

• *Xenopus* embryos at stage 10.5 were immersed in 4 different concentrations of malathion (20 embryos per concentration) from 0 mg/L (control) to 3.5 mg/L.

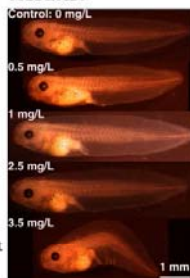
• Embryos were left in the dark to develop and were checked daily. Any tadpoles that died were removed.

• After 72 hours, tadpoles were anesthetized, fixed in 1% glutaraldehyde for 24 hours, and then rinsed four times in PBS. Photographs were taken using Adobe Photoshop and measurements (angle and length) were made using ImageJ.

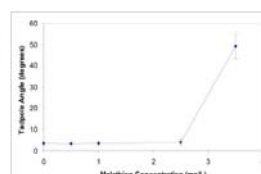
Body axis angle (θ) was measured with reference to 0° (straight) from the center of embryo eye to the point of bend and through the tip of the tail.



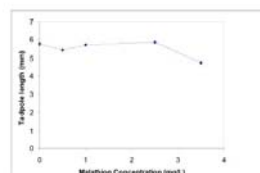
Results:



Malathion caused deformation in tadpoles exposed to concentrations of 3.5 mg/L. Note the bent body axis in tadpoles exposed to 3.5 mg/L malathion as opposed to the straight body axis of tadpoles exposed to lesser concentrations.



Malathion caused increased body axis angle in concentrations of 3.5 mg/L (p -value = 3.28×10^{-36}). Angles stayed constant with lesser concentrations (p -value = .98). Error bars=standard error at 95% confidence.



Tadpoles exposed to 3.5 mg/L malathion were shorter than tadpoles exposed to lesser concentrations (p -value = 2.96×10^{-14}). Error bars=standard error at 95% confidence.

Summary and Conclusions:

• The angles and lengths of tadpoles exposed to less than 3.5 mg/L were similar to the controls.

• *Xenopus* embryos exposed to 3.5 mg/L malathion showed an average body axis angle of 50° and an average length of 4.73 mm. This difference is likely due to deformity in notochord.

3.5 mg/L malathion inhibit *Xenopus* embryo development by increasing angle and decreasing length of embryo body axis.

References:

- ¹<http://micro.magnet.fsu.edu/pesticides/pages/malathion.html>
- ²Snowder & Chambers (1993) Toxicol. Appl. Pharmacol. 121: 210-16.

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